AD_____

AWARD NUMBER: W81XWH-06-1-0533

TITLE: Inflammatory markers and breast cancer risk

PRINCIPAL INVESTIGATOR: Brenda Diergaarde, PhD

CONTRACTING ORGANIZATION:
University of Pittsburgh
Pittsburgh, PA 15260

REPORT DATE: July 2009

TYPE OF REPORT: Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2009	Annual	1 Jul 2008 - 30 Jun 2009

4. TITLE AND SUBTITLE
Inflammatory markers and breast cancer risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0533

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Brenda Diergaarde, PhD, Eleanor Feingold, PhD, Russell Tracy, PhD, and

Robert Ferrell, PhD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh

Pittsburgh, PA 15260

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Mammographic breast density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts. Information on the etiology of breast density is currently limited. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between serum cytokine levels, genetic variation in cytokine genes, and breast density using data and samples from the Mammograms and Masses Study. This report provides information on the progress made during the third year of the grant. MAMS study participants were genotyped for common single nucleotide polymorphisms (SNPs) in or near *IL6*, *IL6R*, *IL6ST*, *TNF-alpha*, *TNFRSF1A*, and *TNFRSF1B* using MassARRAY iPLEX Gold (Sequenom). Analyses of the genotype data are ongoing. Preliminary results suggest that, in healthy postmenopausal women, common variation in *IL6R* and *IL6ST* is associated with percentage breast density. None of the evaluated SNPs in *IL6* and *TNF-alpha* were significantly associated with percentage breast density in this study population.

15. SUBJECT TERMS
Breast cancer; breast density; cytokines; genetic variation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

INTRODUCTION

Mammographic breast density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts (1, 2). Information on the etiology of breast density is currently limited, and the biological mechanism by which mammographic breast density is associated with breast cancer risk is unclear. Various evidence suggest that exposure to sex hormones, estrogens in particular, may be an important factor in breast density. Changes in density have been observed in response to hormone replacement therapy use and use of tamoxifen (3, 4). Pro-inflammatory cytokines, specifically tumor necrosis factor (TNF)-α and interleukin (IL)-6, have emerged as critical regulators of estrogen synthesis in breast tissues (5), and may so affect breast density and breast cancer risk. In line with this, polymorphisms in *IL6* have been found associated with breast cancer risk and to modify the association between estrogen and aspirin and breast cancer risk (6). To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between serum cytokine levels, genetic variation in cytokine genes, and breast density. Existing data and banked specimens from women who participated in a recently completed, cross-sectional study on hormones and breast density, the Mammograms and Masses Study (MAMS), are used.

BODY

- **Cytokine serum levels and mammographic breast density**

As reported in our previous annual report, serum levels of IL-6, soluble IL-6R and TNF-α were measured for all 722 study participants. Our preliminary results suggested that there is no significant association between IL-6 and TNF-α serum levels and breast density. Due to issues with the ELISA assays (results not reproducible) we had been unable to measure soluble TNF-R1 and TNF-R1 levels. Since then we have investigated other assays, and we are currently in the process of measuring soluble TNF-R1 and TNF-R1 levels (in duplicate) using Luminex Technology (Cytokine Human TNF-RI Single-Plex kit and Cytokine Human TNF-RII Single-Plex kit, respectively) in the Immunologic Monitoring Laboratory of the University of Pittsburgh Cancer Institute (Pittsburgh, PA). Both sTNF-RI and sTNF-RII are known to modulate TNF-α activity by decreasing TNF- α levels, and for completeness we plan to publish the results for these receptors together with those for IL-6 and TNF-α.

- **Genetic variation and mammographic breast density**

DNA was extracted from buffy coat samples and plates were prepared for genotyping in Dr. Robert Ferrell's laboratory in the Department of Human Genetics of the University of Pittsburgh (Pittsburgh, PA).

Study participants were genotyped for, in total, 45 single nucleotide polymorphisms (SNPs) located in or near *IL6* (9 SNPs), *IL6R* (12 SNPs), *IL6ST* (7 SNPs), *TNF-α* (1 SNP), *TNFRSF1A*

(7 SNPs), and *TNFRSF1B* (9 SNPs). The *IL6ST* gene was included because IL-6 acts by binding to IL-6R which must associate with gp130 (coded for by *IL6ST*) in order for signal transduction to occur. Putative functional SNPs were selected using public databases such as the Genome Variation Server (http://gvs.gs.washington.edu/GVS/) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), and literature search. Additionally, for each gene except *TNF-α*, tagSNPs capturing common variants in the gene region were selected using data from the International HapMap project (www.hapmap.org; CEU population) and Haploview's Tagger (7, 8) [criteria used: minor allele frequency (MAF)>0.05 and pairwise correlation $r^2 \geq 0.80$]. All genotyping was performed at the University of Pittsburgh Genomics and Proteomics Core Laboratories (Pittsburgh, PA). SNP rs1800629 (*TNF-α* -308 G/A), was assessed using TaqMan (Assay ID: C___7514879_10; Applied Biosystems, Foster City, CA). All other SNPs were genotyped using MassARRAY® iPLEX Gold (Sequenom, Inc., San Diego, CA); the SNP specific and mass extend oligonucleotides, and assays were designed using Sequenom RealSNP (www.realsnp.com) and MassARRAY Assay Design version 3.1 (Sequenom, Inc., San Diego, CA). Sample duplicates (N=36) were included to monitor genotyping quality; no discrepant genotypes were observed. Analyses were restricted to women with genotyping call rates of ≥90%.

The current preliminary analyses are restricted to Caucasian postmenopausal women with a negative routine screening mammogram, no previous cancer, available mammogram and questionnaire data, and genotyping call rates of ≥90%; N=369. Selected characteristics of the study population are presented in Table 1. The mean age of the study participants was 62.1 years, the mean body mass index (BMI) was 28.1 kg/m^{2}m, and the average percentage breast density was 30.2%.

Deviation from Hardy-Weinberg equilibrium was assessed with the Chi-square goodness-of-fit test. With the exception of rs2228576 in *TNFRSF1A* (P=0.0002) and rs653667 in *TNFRSF1B* (P=0.0013), all SNPs were in Hardy Weinberg equilibrium. Linear regression models (GLM procedure in SAS) were used to examine the relationship between each SNP and percentage breast density. Because the number of rare-allele homozygotes in some cases was relatively small, heterozygotes and rare-allele homozygotes were combined in the analyses (common allele homozygotes were used as the reference group). Percentage breast density was square-root transformed to normalize the distribution. For ease of interpretation, the presented means were transformed back to the original scale. To determine if there was a linear trend with increasing variant alleles, P values were also calculated with a linear regression model based on the number of copies of rare alleles (0, 1, 2). All models were adjusted for age (continuous), BMI (continuous), hormone therapy use (never, past, current), NSAID use (no, yes), pregnancy for at least 6 months (no, yes), and previous biopsy (no, yes). All significance tests were two-sided; P values <0.05 were considered statistically significant. All analyses were performed with use of the SAS® statistical software package (SAS version 9.2, SAS Institute Inc., Cary, NC). Results are presented in Table 2.

Table 1: Selected characteristics of the study population

	All study participants N_{total}=369 N(%)
Age (in years):	
- younger than 50	3 (0.8)
- 50-59	164 (44.4)
- 60-69	128 (34.7)
- 70 or older	74 (20.1)
Body mass index (kg/m^2):	
- normal (less than 25.0)	124 (33.6)
- overweight (25.0-<30.0)	130 (35.2)
- obese (30.0 or more)	115 (31.2)
Smoking:	
- never	211 (57.2)
- former	138 (37.4)
- current	20 (5.4)
Current NSAID use	182 (49.3)
First-degree relative with breast cancer[a]	55 (15.0)
Previous biopsy	55 (14.9)
Older than 12 years of age at menarche	184 (49.9)
Ever been pregnant	308 (83.5)
Age at first pregnancy lasting ≥6 months (in years):	
- younger than 20	28 (7.6)
- 20-24	131 (35.5)
- 25-29	88 (23.9)
- 30 or older	50 (13.6)
- never pregnant/no pregnancies ≥6 months	72 (19.5)
Younger than 50 years of age at menopause[b]	147 (40.6)
Type of menopause[c]:	
- natural menopause	263 (74.1)
- hysterectomy without oophorectomy	41 (11.6)
- hysterectomy with uni- or bilateral oophorectomy	51 (14.4)
Postmenopausal hormone therapy use:	
- never	133 (36.0)
- former	186 (50.4)
- current (within previous 3 months)	50 (13.6)

[a] Variable not available for 3 participants; [b] Variable not available for 7 participants; [c] Variable not available for 14 participants.

Table 2: Mean percentage mammographic density by genotype

GENE	SNP	Genotype	Mean % density[a]	*P*[b]	P_{TREND}
IL6					
	rs1800795	G/G (N=132)	24.94	0.23	0.57
		G/C (N=165) or C/C (N=56)	27.22		
	rs2069827	G/G (N=298)	26.81	0.33	0.4
		G/T (N=50) or T/T (N=4)	24.33		
	rs7801617	G/G (N=268)	26.73	0.43	0.43
		G/A (N=81) or A/A (N=10)	25.12		
	rs2069840	C/C (N=163)	26.86	0.63	0.65
		G/C (N=160) or G/G (N=32)	25.98		
	rs2069861	C/C (N=295)	26.2	0.72	0.85
		C/T (N=61) or T/T (N=3)	27.06		
	rs12700386	C/C (N=245)	26.56	0.75	0.91
		G/C (N=100) or G/G (N=11)	25.93		
	rs2069837	A/A (N=305)	26.42	0.78	0.67
		G/A (N=51) or G/G (N=3)	25.72		
	rs7805828	G/G (N=140)	26.72	0.84	0.8
		A/G (N=160) or A/A (N=53)	26.33		
	rs2069860	A/A (N=354)	26.33	0.89	0.89
		A/T (N=5)	25.23		
IL6R					
	rs6427627	T/T (N=138)	23.57	0.01	0.22
		C/T (N=170) or C/C(N=47)	28.39		
	rs11265608	G/G (N=299)	25.45	0.03	0.02
		A/G (N=56) or A/A (N=4)	30.88		
	rs1386821	A/A (N=230)	25.04	0.05	0.16
		C/A (N=113) or C/C (N=16)	28.76		
	rs4240872	T/T (N=215)	27.57	0.11	0.23
		C/T (N=127) or C/C (N=17)	24.58		
	rs10159236	C/C (N=234)	25.58	0.25	0.24
		C/A (N=105) or A/A (N=16)	27.84		
	rs4072391	C/C (N=242)	26.88	0.37	0.57
		C/T (N=104) or T/T (N=13)	25.17		
	rs2228145	A/A (N=117)	27.42	0.4	0.34
		C/A (N=187) or C/C (N=48)	25.8		
	rs11265622	G/G (N=143)	27.11	0.47	0.79
		A/G (N=156) or A/A (N=60)	25.8		
	rs2054855	C/C(N=256)	26.06	0.65	0.76
		C/T (N=92) or T/T (N=11)	26.98		
	rs6684439	C/C (N=124)	26.96	0.77	0.51
		C/T (N=178) or T/T (N=47)	26.41		
	rs4845618	T/T (N=125)	26.09	0.85	0.7
		G/T (N=178) or G/G (N=56)	26.44		
	rs4601580	A/A (N=106)	26.24	0.99	0.87
		A/T (N=189) or T/T (N=62)	26.27		

Table 2 cont.

GENE	SNP	Genotype	Mean % density[a]	*P*[b]	P_{TREND}
IL6ST					
	rs11574780	A/A (N=311)	27.09	0.03	0.03
		A/G (N=34)	20.67		
	rs10940495	A/A (N=189)	27.66	0.13	0.32
		G/A (N=150) or G/G (N=20)	24.93		
	rs1063560	C/C (N=341)	26.64	0.14	0.14
		C/G (N=15)	20.35		
	rs10471417	A/A (N=165)	25.54	0.4	0.58
		C/A (N=147) or C/C (N=43)	27.08		
	rs6870870	C/C (N=136)	27.19	0.51	0.42
		C/A (N=168) or A/A (N=51)	25.93		
	rs2228043	C/C (N=289)	26.35	0.99	0.82
		C/G (N=63) or G/G (N=5)	26.39		
	rs1900173	T/T (N=309)	26.38	1	0.77
		T/A (N=37) or A/A (N=2)	26.37		
TNFRSF1A					
	rs4149584	G/G(N=338)	26.13	0.26	0.26
		G/A (N=18)	30.95		
	rs4149577	T/T (N=94)	25.31	0.49	0.98
		T/C (N=179) or C/C (N=83)	26.74		
	rs4149579	G/G (N=313)	26.16	0.65	0.65
		G/A (N=45) or A/A (N=1)	27.4		
	rs2228576	G/G (N=171)	26.85	0.71	0.96
		G/A (N=162) or A/A (N=11)	26.15		
	rs4149570	G/G (N=131)	26.06	0.79	0.8
		G/T (N=167) or T/T (N=52)	26.57		
	rs11064145	T/T (N=103)	26.02	0.82	0.85
		G/T (N=180) or G/G (N=70)	26.48		
	rs4149578	G/G (N=300)	26.31	0.83	0.65
		G/A (N=51) or A/A (N=4)	26.87		
TNFRSF1B					
	rs652284	T/T (N=99)	23.71	0.06	0.34
		T/C (N=182) or C/C (N=78)	27.39		
	rs1201157	C/C (N=139)	27.68	0.25	0.5
		C/T (N=165) or T/T (N=52)	25.53		
	rs5746016	C/C (N=318)	26.08	0.39	0.38
		T/C (N=34) or T/T (N=1)	28.78		
	rs590977	A/A (N=238)	26.89	0.42	0.44
		C/A (N=115) or C/C (N=15)	25.38		
	rs5746001	G/G (N=236)	26.8	0.51	0.49
		G/A (N=105) or A/A (N=15)	25.51		
	rs653667	A/A (N=88)	27.18	0.62	0.31
		C/A (N=207) or C/C (N=59)	26.14		
	rs683240	T/T (N=189)	26.66	0.65	0.65
		C/T (N=147) or C/C (N=22)	25.85		
	rs1061622	T/T (N=193)	26.66	0.78	0.73
		G/T (N=140) or G/G (N=22)	26.15		
	rs816060	T/T (N=102)	26.58	0.81	0.33
		C/T (N=178) or C/C (N=77)	26.09		

Table 2 cont.					
GENE	**SNP**	**Genotype**	**Mean % density[a]**	***P*[b]**	***P*$_{TREND}$**
TNF-α					
	rs1800629	G/G (N=278)	26.85	0.29	0.31
		A/G (N=77) or A/A (N=4)	24.6		

[a] Mean percentage breast density adjusted for age, BMI, hormone therapy use, NSAID use, pregnancy for at least 6 months and previous biopsy.
[b] *P* value for difference in mean percentage breast density adjusted for age, BMI, hormone therapy use, NSAID use, pregnancy for at least 6 months and previous biopsy.

None of the evaluated SNPs in *IL6* and *TNF-α* were significantly associated with percentage mammographic density in our study population. However, two SNPs in *IL6R*, rs11265608 and rs64227627, and one in *IL6-ST*, rs11574780, were statistically significantly associated with percentage mammographic density. For both rs11265608 and rs64227627, mean percentage mammographic density was significantly higher among women with at least one rare allele than among women homozygous for the common allele (*P*=0.01 and *P*=0.03, respectively). For rs11574780, mean percentage mammographic density was significantly higher among women homozygous for the common allele (*P*=0.03).

Interactions with BMI, NSAID use and hormone therapy use have not yet been assessed but will be investigated.

KEY RESEARCH ACCOMPLISHMENTS

Progress was made during the third year of this grant. However, not all work was completed during the approved project period due to the grant not having a Principal Investigator and not being able to use grant money from the time Dr. Modugno left until January 2008 when Dr. Diergaarde officially took over. Additional time beyond the established expiration date to ensure adequate completion of the originally approved project was requested and granted.

The preliminary results from the SNP data suggest that suggest that common variation in *IL6R* and *IL6ST* is associated with percentage mammographic density in healthy Caucasian postmenopausal women.

REPORTABLE OUTCOMES

An abstract on the relation between common genetic variants in *IL6*, *IL6R*, *IL6ST*, *TNF*, *TNFRSF1A* and *TNFRSF1B*, and mammographic breast density has been submitted to the 2010 American Society of Preventive Oncology (ASPO) meeting. A manuscript on this topic is in preparation as is a manuscript on the relation between cytokine levels and mammographic breast density (regarding the latter, we are currently waiting on the measurement of sTNF-RI and sTNF-RII, see above).

CONCLUSION

Polymorphisms in *IL6*, *TNF-α* and the genes that code for their receptors may alter exposure to estrogens and so affect mammographic breast density. In line with this, our preliminary results suggest that common variation in *IL6R* and *IL6ST* is associated with percentage mammographic density in healthy Caucasian postmenopausal women. Identification of the genes (and within the genes the functional polymorphisms) that affect mammographic breast density will likely provide insights into the biology of the breast and may identify potential targets for breast cancer (chemo)prevention.

REFERENCES

1. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2006;15:1159-1169.
2. Vachon CM, Brandt KR, Ghosh K, *et al*. Mammographic breast density as general marker of breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 2007;16:43-49.
3. Rutter CM, Madelson MT, Laya MB, Segers DJ, Taplin S. Changes in breast density associated with initiation, discontinuation, and continuing use of hormone replacement therapy. *JAMA* 2001;285:171-176.
4. Cuzick J, Warwick J, Pinney E, Warren RM, Duffy SW. Tamoxifen and breast density in women at increased risk of breast cancer. *J Natl Cancer Inst* 2004;96:621-6218.
5. Purohit A, Newman SP, Reed MJ. The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer. *Breast Cancer Res* 2002;4:65-69.
6. Slattery ML, Curtin K, Baumgartner R, *et al*. Il6, aspirin, nonsteroidal anti-inflammatory drugs, and breast cancer risk in women living in the southwestern United States. *Cancer Epidemiol Biomarkers Prev* 2007;16:747-755.
7. Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263-265.
8. Bakker de PIW, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D Efficiency and power in genetic association studies. *Nature Genetics* 2005;37:1217-1223

APPENDICES

None